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Effect of Soyasaponin Fractions on Human Colon Cancer Cells

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By

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Abstract

Group B saponins, the predominant form of saponins in heat-treated soy products, have been shown to possess hypocholesterolemic, antimutagenic, and anticarcinogenic properties. Previous studies have evaluated crude mixtures of soyasaponins, but studies evaluating a single purified soyasaponin as an anticarcinogenic agent are limited. The *goal* of this study is to examine the effects of purified soyasaponins I and III as well as their aglycone form, soyasapogenol B, as anticarcinogenic agents on the human colon adenocarcinoma cell line Caco-2. Experiments were conducted to determine the effects of purified soyasaponins on cell proliferation, Protein Kinase C (PKC) activity, and cell morphology in cultures of Caco-2 cells. Treatment of cells with soyasaponins I and III at concentrations of 300–900 ppm significantly reduced viable cell numbers after 48 and 72 hours of exposure by 10-35% ($p<0.05$). Soyasapogenol B at a concentration of 100 and 150 ppm significantly reduced viable cell numbers after 24 hours by 15 and 62%, respectively ($p<0.05$). Cell morphology changes demonstrated that as concentrations and lipophilicity of soyasaponins increased, cell membranes became rougher and more irregular. Treatment of cells for 72 hours significantly reduced the amount of PKC activity by 9-40% ($p<0.05$). Results indicate that purified soyasaponins I, III and soyasapogenol B, at physiologically relevant doses, can suppress Caco-2 colon cancer cell proliferation. These findings suggest that purified group B soyasaponins and their final metabolite soyasapogenol B may be a colon-cancer suppressive component of soy that warrants further examination as a potential nutraceutical or functional food.

This thesis is approved for recommendation
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Table of Contents

List of Tables and Figures

Tables

List of Figures

Chapter I. Introduction

Colorectal Cancer (CRC) is the third most commonly diagnosed cancer and is the second leading cause of mortality in the U.S., causing an estimated 49,920 deaths in 2009 in addition to the 146,970 new cases that were also reported [1]. According to the American Cancer Society, the lifetime risk of being diagnosed with cancer of the colon or rectum is 5.5% for men and 5.1% for women in the US. Remarkably, only 20-25% of CRC cases occur among individuals with a family history of CRC or a predisposing condition and the remaining 75% of cases occur in people without these risk factors [2]. Prevalence of CRC has been declining in the U.S. since 1998, however, worldwide incidence and deaths from colon cancer are generally increasing, especially in the developed world and urban areas of developing countries [3]. North America, Australia/New Zealand, Western and Eastern Europe have the highest colon cancer rates. These areas account for over 63% of incidence worldwide [3]. In what used to be considered low risk areas like Central and South America, Asia, and Africa, rates are beginning to increase as well [4]. Because of these large geographic differences, it is likely that a number of factors affect the likelihood of developing colon cancer; among them being heredity, sex, age, race, pre-existing conditions, lifestyle, and diet.

The **goal** of this study was to evaluate the effects of the predominant soyasaponin in heat treated soy products (soyasaponin I) and its two predominant metabolites (soyasaponin III and soyasapogenol B) on human colon cancer cells. The **hypothesis** of this study is that purified soyasaponin I and its metabolites are more effective at suppressing colon cancer cell growth than crude mixtures of soyasaponins. The **objectives** of this study were 1) to investigate what soyasaponins are most effective at inhibiting colon cancer cell growth and 2) to determine at what concentration they are most potent. To accomplish these objectives, cell viability and

protein kinase C (PKC) were measured and cell morphology changes were observed. The effects of soy and other topics important to the development of colon cancer will be addressed in the following review.

Chapter II. Review of Literature

Colon Cancer

Colon cancer is the product of both environmental and hereditary factors that have yet to be completely identified or understood. Data from numerous epidemiological studies indicate diet and soy consumption as important factors contributing to the risk and development of certain types of cancer, particularly breast, prostate, and colon. Investigations into the relationship between cancer and soy intake has led scientists to conclude that phytochemicals within soy are responsible for these effects, and that the mechanisms by which different phytochemicals exert their effects are as varied as the phytochemicals themselves.

Pathogenesis of Colon Cancer

The development of colon cancer is a multistep process that is a consequence of a closely tied, but not fully understood, interaction between genetic and environmental factors [5]. A defining characteristic of colon cancer is the step-wise progression from normal colonic tissue to the malignant cell growth that is associated with chronological molecular deviations leading to tumor development [6]. It was discovered in the 1990s that the accumulation of activated oncogenes and the inactivation of tumor-suppressor genes that the colorectal epithelium progresses from a normal phenotype to one that is hyperproliferative and malignant in nature [5].

Sporadic CRC is one of the most common malignancies worldwide; because of this, there has been much research done in order to elucidate the underlying causes. This has revealed complex processes that lend to the generation of the required mutations and identification of various groups of target genes that govern the biological behaviors which are seen in tumor cells [7].

As mentioned above, colon cancer development is a multi-stage process in which altered cells progress to precancerous lesions such as adenomatous polyps, that if given enough time, will develop into carcinomas. The gastrointestinal epithelium is a complex microenvironment that is composed of at least four different interconnected cell types. In addition to other cell types, absorptive enterocytes, goblet cells, Paneth cells, and enteroendocrine cells are characterized by a tightly-regulated succession of cell proliferation, maturation, differentiation, and apoptosis [7]. The genetic model proposed by Fearon and Vogelstein [5] indicates that a hyperproliferative colonic epithelium is the first step towards colon cancer development. Populations that are at high risk (advanced age, genetically predisposed, presence of adenomatous polyps, or familial adenomatous polyposi [FAP]) have been shown to have increased mucosal cell proliferation throughout their colon [7]. Gene mutations and/or deletions accompany the promotion of a hyperproliferative normal crypt to an adenoma. It is during this initiation stage that the gatekeeper genes which are responsible for the control of cell proliferation, differentiation, migration, and apoptosis within the colonic mucosa are altered [8-11]. The transformation to a cancerous state is often associated with the loss of several more genes including those that prevent cells with damaged DNA from progressing to the S-phase of the cell cycle, which thereby makes them responsible for the repair of DNA before allowing another round of cell division [12-14].

Increased proliferation is seen in adenomas when compared to normal epithelium, and in addition to this, a shift in the proliferation toward the luminal surface of the epithelium occurs in adenomas as well as aberrant crypt foci (ACF) [7, 15]. Indicators of the increased colorectal crypt cell proliferation and of the expansion of the proliferative zone toward the luminal surface

of the crypts have been identified. These indicators are of interest because they are biomarkers of future risk for the development of colon tumors [5, 16, 17].

ACF are induced by colon specific carcinogens in a dose dependant manner, evolve from one altered crypt, and exhibit preneoplastic features (dysplasia, abnormal cell proliferation patterns, and various gene mutations) [18]. ACF have shown to be larger than normal crypts, show increased branching and proliferation, and reportedly contain cells which demonstrate a variety of changes that indicate they are at an early stage in the process of cancer development [19-21]. In addition to rodent models, ACF have been described in human colonic mucosa as well [22-25]. Perhaps more importantly is the fact that they are predictors of tumor outcome and are a valuable tool for quantifying risk [15, 18, 26]. It is because of these characteristics that ACF are of concern to researchers interested in the early morphological changes which take place in the development of tumors or adenocarcinomas.

Epidemiology

Numerous studies and reports have shown that cancer is a widespread disease, and according to the World Health Organization (WHO), was responsible for 7.9 million deaths in 2007. Whereas the universality of cancer is not disputed, the distribution of certain cancer types and target organs is distributed unequally among different countries, those who live in urban areas as compared to rural areas, people of different ethnicities, race, socioeconomic status, and sex [27]. Such variations are inevitably due to genetic variations, environmental factors, and diet.

According to the International Agency for Research on Cancer, worldwide rates of CRC have variances of up to 25-fold, the highest rates being found in the developed world and the

lowest in India. However, even within this one country, there are differences in CRC incidence between populations, such as the increased rates of the Westernized Parsi and the strictly vegetarian Janists [28]. Supporting evidence for this trend can be seen in migrant studies, such as Japan to USA and Eastern Europe to North America. When studying the change in CRC rates among immigrants, when populations moved from low- to high-risk areas, the connection to environmental factors becomes quite evident; the rate of CRC increases rapidly, indicating that dietary as well as other environmental factors contribute a great deal to risk development [29, 30]. Proof of this can be seen in Japanese individuals who were born in the USA now having higher rates than those of US whites, and the rates of colon cancer of Japanese living in Hawaii and Los Angeles are among the highest in the world [31]. Migrant studies have also shown incidence rates of migrants and their descendants reaching that of the host country, sometimes even within one generation [4]. These studies demonstrate how a change from a plant-based diet to a Westernized diet affects the etiology of colorectal malignancies and shows a clear relationship between diet and cancer risk, thereby making it of considerable interest to those in the medical field as well as nutrition researchers.

Diet and Colon Cancer Relationship

Genetics and environmental factors can only explain so much, considering that genetic factors alone are only believed to explain roughly 5% of all cancers [32]. With that in mind, it is estimated that approximately 66-75% of CRC could be prevented by proper diet and lifestyle changes [4]. The role of diet in cancer prevention has been documented, but the exact dietary components that have greatest effect on risk have been difficult to pinpoint. The difficulty in showing direct relationships between dietary components and their corresponding cancer risk is

due in large part to inconsistent data from community- and population-based studies [32]. Factors contributing to these inconsistencies include the lack of an accurate measurement of types of dietary fat in the foods of the population under investigation as well as inconsistent questionnaires intended to quantify fruit and vegetable intake [17, 33]. Evidence from numerous cohort and case-control studies has shown that there is an inverse relationship between diets rich in fruits and vegetables and cancer risk. Different factors responsible for this finding include: fiber intake, micronutrients (carotenoids, ascorbate, and folate among others), and phytochemicals [17].

Although no strong correlation between any one particular food and the increased risk of colon cancer can be made, there have been many epidemiological studies which conclude that the consumption of soy foods may contribute to lower rates of various cancers, namely colon, breast, and prostate cancer in countries such as China and Japan [34-36]. Japan has been heavily studied for their plant-based diet, specifically their consumption of soy and soy products. There has been considerable interest in the soy consumption of this country because of their lower-than-average disease rates and the belief that soy has health benefits that go beyond the nutrients it provides. Several epidemiological studies [37-40] have been conducted in order to determine soy intake and related disease risk or incidence of the study populations. Soy is a rich source of phytochemicals such as isoflavones and saponins. Phytochemicals are non-nutrient secondary metabolites of plants that are biologically active. Of which, some have been shown to possess various activities that are beneficial to health when they are consumed as part of the diet. Because isoflavones have been so intensely investigated for their potential health benefits and because saponins are a major group of phytochemicals in soy, this review and subsequent research project will focus on soyasaponins.

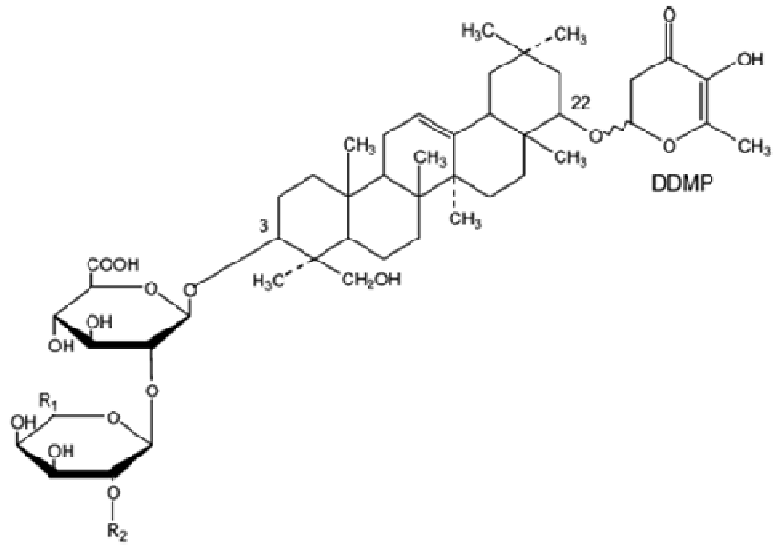
Soyasaponins

Saponins are a group of amphiphilic and structurally complex glycosides that consist of one or more oligosaccharide moieties linked to a triterpenoid or steroidal aglycone ring structure (sapogenol). These can be found in a variety of plants such as ginseng, Quillaja, Gypsophila, and soybeans. Their surface-active properties, which are a result of their hydrophilic sugars and lipophilic sapogenol, are the source of the name saponin, which comes from the Latin word for soap "sapo" [41]. It is important to note that the bioactivity of saponins vary depending on their source and chemical make-up [42, 43], which includes variances between ring structures as well as number and type of carbohydrate moieties.

Chemistry of Soyasaponins

Soyasaponins, as their name implies, are saponins from soybean. Soyasaponins vary in the composition of their soyasapogenol as well as the number, type, and point(s) of attachment of their sugar moieties to the soyasapogenol (**Figure 1**). Originally, it was thought there were up to five different soyasapogenols, however, as research advanced it was determined that soyasapogenols C, D, and E were artifacts of hydrolysis and that there are two specific pentacyclic triterpenoid sapogenols to which the sugar moieties attach: soyasapogenol A and soyasapogenol B [44-47] (**Figure 2**). A number of different sugar moieties exist, including L-arabinose and L-rhamnose which are linked α-glycosidically, and D-glucose, D-xylose, D-galactose, and D-glucuronic acid which are β-glycosidically-linked [41]. These sugar moieties are attached to position 3 of the triterpenoid aglycone by an ether linkage. Because group B soyasaponins are the predominant form in soy and soy products, they will be the focus of this literature review and subsequent research.

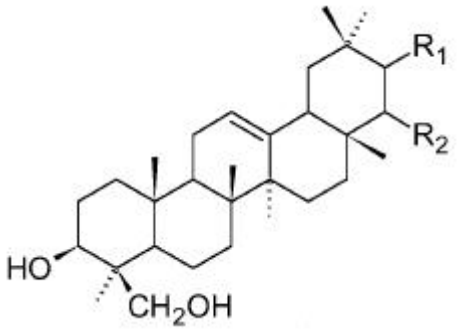
Figure 1. Soyasaponin structure and nomenclature, adapted from Kudou *et al.* [49]



DDMP	Non-DDMP	R_1	R_2
αg	V	CH_2OH	α-L-Rhamnose
βg	I	CH_2OH	B-D-Glucose
βa	II	H	α-L-Rhamnose
γg	III	CH_2OH	H
γa	IV	H	H

It appears that the group B soyasaponins (αg, βg, βa, γg and γa) exist in the intact plant tissue as conjugates of 2,3-dihydro-2,5- dihydroxy-6-methyl-4H-pyran-4-one (DDMP) at the 22 hydroxyl position and are considered to be the genuine form of saponin [48]. The bond connecting the DDMP molecule to the soyasaponin is easily degraded, resulting in the formation of the non-DDMP group B soyasaponins (V, I, II, III, and IV), which explains their predominance in heat treated soy products [48, 49]. The degradation of the DDMP-conjugated soyasaponin yields maltol and the corresponding non-DDMP-conjugated soyasaponin. This means DDMP group B soyasaponin αg → non-DDMP group B soyasaponin V, βg → I, βa→II, γg→III, and γa→IV.

Figure 2. Soyasapogenol A and B chemical structures



Aglycone	R_1	R_2
Sapogenol A	OH	OH
Sapogenol B	H	OH

Bioactivity of Soyasaponins

Evidence from numerous studies indicates that soyasaponins have a wide variety of physiological effects. Antioxidant activity of soyasaponins, namely the DDMP-conjugates has been evaluated in their ability to act as free radical scavengers because of their ability to donate an electron from its double bond moiety [50-53]. The *in vivo* hypocholesterolemic effects of soyasaponins have been evaluated as well [54, 55]. Other *in vitro* studies have demonstrated hepatoprotective [56-58], antiviral [59-61], anti-inflammatory [61], and immune-enhancing properties [62]. Because of their unique structure and amphiphilic nature, soyasaponins have been studied for a wide variety of biological effects; however, much of the interest in soyasaponins lies in their anti-carcinogenic properties.

Anti-Carcinogenic Activity in Tissue Culture and Animals

There have been multiple *in vitro* studies [63-75] and one *in vivo* study [76] conducted to investigate the anti-mutagenic or anti-carcinogenic properties of soyasaponins. It is from these studies that we have gained more of an understanding as to how soyasaponins may suppress the growth of cancer cells. Through *in vitro* studies, the effect a known compound has on a specific

type of cell and cell process (i.e. apoptosis, differentiation, and proliferation) can be closely monitored and the molecular pathway can be elucidated, additionally, the overall effect on health and organ systems can be observed in animal studies.

In Vitro Studies

There have been numerous studies which utilized different soyasaponins and soyasapogenols at various concentrations and have displayed anticarcinogenic properties in several different carcinoma cell lines [63-66, 68, 71-75, 77]. The results of these studies are summarized in **Table 1**. From the studies listed, it is apparent that there are several factors that contribute to the observed effects of soyasaponins and their sapogenol on cancer cell lines. The first of these factors is the testing material itself. The majority of these studies utilized a crude mixture of all group-A and group-B soyasaponins or just group-B soyasaponins, whereas others used semi-purified individual soyasaponins. The use of crude extracts makes it difficult to match a result observed in the cells to a molecular action of a specific soyasaponin. Because of this, studies with purified soyasaponins are very important for identifying how purified soyasaponins affect bioactivity and the mechanisms involved in cell growth and viability. The second factor is the dose. Concentrations ranging from 6.25-2400 ppm have been used in various studies yielding different degrees and types of cell suppression. The third factor affecting results is the incubation time. Most studies monitor cell growth and viability at 24, 48, and 72 h, however this can vary from as little as 4 hours [74] to as long as 5 days [63]. Such variances are bound to yield different results.

Table 1. Summary of anticarcinogenic activities of soyasaponins and their sapogenol

Type of Saponin	Cell Line	Concentration	Observation	Mechanism	Reference
Group-B soyasaponin mixture	HCT-15	150-600 ppm	Dose-dependent reduction in cell growth and viability after 48 h.	Formation of cytoplasmic vesicles and deformation of plasma and nuclear membrane.	[66]
Group-B soyasaponin mixture	HCT-15	150-600 ppm	Dose-dependent reduction in growth and viability after 48 h. Growth completely inhibited at 24 h with 300 and 600 ppm.	Unknown, possibly due to saponin-cell membrane interactions	[67]
Group-B soyasaponin mixture	HT-29	150-600 ppm	Dose-dependent reduction in cell growth when maintained for 72 h.	Suppression of PKC activity and induction of differentiation	[65]
Soyasaponin I (89% pure) Soyasaponin III (86% pure) Soyasapogenol B (93% pure)	HT-29	6.25, 12.5, 25, 50 ppm	Saponin I had no inhibitory effect on cell growth Saponin III suppresses growth at 50 ppm after 72 h. Sapogenol B shows dose-dependent suppression from 6-50 ppm after 72 h.	The suppression of cell growth is intensified with the increase in lipophilicity. (Sapogenol B > Saponin III > Saponin I)	[73]
Group-B soyasaponin mixture	HT-29	150-600 ppm	Dose-dependent reduction in cell growth at all concentrations after 72 h.	Down regulation of COX-2 and PKC expression	[64]

Table 1. (continued)

Type of Saponin	Cell Line	Concentration	Observation	Mechanism	Reference
Soyasaponin I Soyasapogenol B	Caco-2	Soyasaponin I 470-2800 ppm Soyasapogenol B 230-1375 ppm	Soyasaponin I had no effect on cell viability Soyasapogenol B significantly reduced cell viability at 230 and 1375 ppm after 4 h	unknown	[74]
Group-B soyasaponin mixture	HCT-15	25-500 ppm	Suppress proliferation dose-dependently and induced macroautophagy at 100 ppm after 24 h	Cells delayed in S-phase of cell cycle.	[71]
Group-B soyasaponin mixture Soyasapogenol B	Caco-2	25-100 ppm	Group-B soyasaponins had no effect on cell growth. Soyasapogenol B significantly reduced cell growth at 25, 50, and 100 ppm	No mechanism offered. However, no negative effects on cell morphology were observed.	[77]
Group-B soyasaponin mixture	HT-29	150-2400 ppm	Decreased cell viability in dose-dependent manner, suppressed PKC activity, and increased AP activity	Induction of cell differentiation at lower concentrations and induction of type II autophagic death at higher concentrations	[63]

Proposed Mechanism of Anticarcinogenic Properties of Soyasaponins and Soyasapogenols *In Vitro*

From published research, it appears as though there may be more than one mechanism of action for the anticarcinogenic effects of soyasaponins. With regard to human colon cancer cell line studies, there have been two widely proposed mechanisms by which soyasaponins elicit their effects. One is the induction of cell differentiation through increases in alkaline phosphatase (AP) and reductions in protein kinase C (PKC) [63-65], and the other is induction of type II autophagic death or macroautophagy [63, 71]. PKC is a marker for cell proliferation and its activity increases as cells undergo the proliferation process. AP is used as a biomarker to indicate differentiation in cells. Studies which monitored the activity of AP and PKC showed that treatment with saponins at lower concentrations were able to induce cell differentiation. Type II autophagic death, or macroautophagy has been observed in two different studies [63, 71] through scanning electron microscopy (SEM), to observe differences in cell morphology, and transmission electron microscopy (TEM), to observe differences in intracellular morphology. In addition to the changes in cellular morphology visible by SEM, under TEM observation, vacuoles appeared in the cells that had been treated with higher concentrations of soyasaponins. This observation was also seen in the study by Ellington *et al.* [71] along with decreased cellular density, the absence of mitochondria, and prominent vacuoles containing degraded cellular material. All of these observations indicate that treatment of cells with group-B soyasaponins does not directly induce apoptosis, but instead induces morphological changes supportive of type II autophagic cell death.

In Vivo Studies

Studies conducted which utilized soy components known to contain both soyasaponins and isoflavones have decreased chemically induced colon carcinogenesis in mice and [78, 79]. However, there have been conflicting results when trying to identify specifically which compounds reduce carcinogenesis in animal models. Soy protein isolate was shown to be protective against azoxymethane (AOM) induced tumors in rats [79]. Isoflavones and soyasaponins were both present in the soy protein, so there can be no definite answer as to which compound was effective in preventing the tumors. Thiagarajan *et al.* [78] demonstrated that soy flour and soy flakes, products known to include soyasaponins and isoflavones, as well as the isoflavone genistein significantly reduced the incidence and multiplicity of ACF in AOM treated rats. They also demonstrated that ethanol washed soy protein concentrate, a product devoid of soyasaponins, did not reduce the incidence or multiplicity of ACF in these rats. Conflicting evidence from a study conducted by Rao *et al.* [80] demonstrated that a diet supplemented with 250 ppm of genistein enhanced carcinogenesis in AOM treated male rats. Because of conflicting results regarding isoflavones, genestin in particular, and because ethanol washed soy does not reduce carcinogenesis in animal models, it appears as though soyasaponins may be responsible for the effects seen in the studies which utilized soy protein or soy flour.

The *in vivo* study mentioned earlier by Koratkar and Rao [76] evaluated the anticarcinogenic effect of a soyasaponin extract containing 8-10 different soyasaponins on an AOM treated CF-1 mouse model. AOM is a colon cancer specific carcinogen intended to initiate cancer in rodents which rarely develop sporadic cancer in nature. One week postinitiation, the mice were placed on a basal AIN-76 diet containing no saponins or one containing 3% soyasaponin by weight. At the end of the 14 week feeding period, the mice were sacrificed and

colons were evaluated for ACF using the method from Bird *et al.* [18]. In this study, the consumption of soyasaponins significantly reduced the incidence of ACF as well as the number of aberrant crypts per focus. The authors suggest that the soyasaponins had undergone microbial hydrolysis of their sugar moieties in the cecum and large intestine, and that because the soyasaponins are not absorbed from the gastrointestinal tract it is likely that they can interact with free or membrane-bound sterols as well as bile acids. This hypothesis agrees with research evaluating the interaction of soyasaponins with bile acids [81-84] and research evaluating the hypocholesterolemic effect of soyasaponins [54, 55].

Proposed Mechanism of Soyasaponins in the Prevention of Colon Cancer *In Vivo*

Within animal models, the ability of soyasaponins and their soyasapogenol to bind with bile acids is widely accepted as the mechanism by which they elicit cholesterol-lowering and anti-colon cancer effects. Bile acids are the major end products of cholesterol metabolism and are formed in the liver. From the liver, they are concentrated and stored in the gallbladder where they can be secreted into the small intestine to act as cholesterol solubilizing agents and aid in lipid digestion and absorption. Under normal conditions, approximately 95% of bile acids passing through the ileum are reabsorbed through the portal vein and return to the liver to be secreted again as part of the enterohepatic circulation.

Primary bile acids which have been transformed to secondary bile acids have been implicated in the carcinogenic process. These amphiphilic molecules are formed in the large intestine by the enzymatic deconjugation and dehydroxylation of primary bile acids by anaerobic colonic bacteria [85]. Secondary bile acids trigger a cascade of events within the colon, each leading to another event potentially resulting in cancer development.

The secondary bile acids, like lithocholic acid, can be co-mutagenic due to their ability to cause DNA strand breaks [86, 87]. Additionally, they are cytotoxic and are disruptive to the colonic mucosal cell membrane integrity [88]. The resulting cell loss stimulates a response by the body to increase cellular proliferation in an attempt to compensate. There is also evidence of a direct stimulatory effect on proliferation mediated by protein kinase C (PKC) activation in the colonic cells [89]. In many cell systems the activation of PKC can lead to the induction of proteins that form transcription factor AP-1 [89]. Bile acids and lipid components in the aqueous fraction of the stool can activate AP-1, whose activation has been associated with promotion of neoplastic transformation [90].

Colon cancer is rarely the result of one single event; typically, it is the final outcome of the interplay between a number of different genetic and environmental variables. One example of this would be the inhibition of apoptosis by deregulation of a number of oncogenes, which could result in colonal expansion. Incidentally, one of the secondary bile acids, deoxycholic acid (DCA) was an effective inducer of apoptosis [88, 91-93]. The enhanced apoptosis by bile acids might be the result of their DNA-damaging properties or their damaging effects on the colonic mucosa.

The anti colon cancer properties of soyasaponins *in vivo* are a result of their ability to bind with primary bile acids and prevent the formation of secondary bile acids. As mentioned before, approximately 95% of bile acids and salts that are secreted into the duodenum are reabsorbed. This allows 2-5% of primary bile acids to escape to the colon and can amount to approximately 20% of the bile acid pool over the course of 6-12 enterohepatic circulations that occur daily. The ability of soyasaponins to bind bile acids within the small intestine and their

soyasapogenol binding to bile acids in the colon could result in a reduction of primary bile acids that are metabolized by the colonic microflora into secondary bile acids.

From the previously mentioned information on bile acid interactions within the colon, it is clear that more insight into the interactions between bile acids, secondary bile acids, and soyasaponins could be valuable in understanding the chemopreventive properties of soyasaponins and their metabolites, which in turn could possibly lead to the development of tumor prevention strategies. These effects seem to be stemming from actions within the lumen of the colon and on the mucosal cell membranes of the colon, it would be pertinent to address the bioavailability, effect of colonic microflora and metabolism of soyasaponins, and possible toxicity issues that may arise from a soyasaponin supplemented experimental diet.

Bioavailability and Metabolism of Soyasaponins

In earlier animal studies, combinations of DDMP and non-DDMP soyasaponins were fed to mice, rats, and chicks [94, 95]. In these studies, saponins were found in the stomachs and small intestines, while only the aglycone sapogenols and a very small amount of saponins were found in the cecum and feces. This suggests that the sapogenols are the primary metabolites.

In a more recent study [75], soyasaponin I was incubated anaerobically with human fecal microflora to investigate how soyasaponins were metabolized and also to identify the metabolites. Over a 48 hr period, it was observed that as soyasaponin I decreased, soyasaponin III increased, and at the end of the 48 hr period the remaining product was soyasapogenol B. This follows a convincing pattern considering soyasaponin I has three sugars, soyasaponin III has two sugars, and soyasapogenol B contains no sugars.

A human feeding study conducted by Hu *et al.* [74] featured an extract containing a mixture of approximately 435 μmol group B soyasaponins which was fed to a group of eight women. Urine and fecal samples were collected and analyzed revealing no soyasaponins in the urine or feces, but sapogenol B was found in the fecal material collected from the subjects. From this study we are able to gain some understanding of the fate of ingested soyasaponins in humans, but more detailed information from *in vivo* conditions is needed.

As part of the previous study by Hu *et al.*, mucosal uptake and transepithelial kinetics of soyasaponin I and soyasapogenol B were measured using the Caco-2 cell monolayer model. The results of this study show that the mucosal transfer rates and apical to basolateral absorptions of soyasaponin I and soyasapogenol B were low. Soyasaponin I showed a saturable and concentration-independent transport and cell-uptake rate, however, soyasapogenol B was taken up by the Caco-2 cells in a concentration-dependent manner. Results of this study confirm the hypothesis of other studies that soyasaponins and their sapogenol have low absorbability in human intestinal cells, are metabolized to the aglycone form by gut microflora, and are excreted in the feces.

Toxicity

An animal toxicity study was performed by MacDonald *et al.*, [77] in which young C57blackJ mice were fed increasing amounts of purified soyasaponins (0-3% by weight) for 4 weeks. The treatment had no negative effects on body weight gain, organ weight, or colon-mucosal parameters. The before mentioned study by Hu *et al.*, [74] demonstrated that soyasaponins have poor absorption in the human body and are metabolized by the microflora within the gastrointestinal tract and are excreted in the feces. Also, the safety of soyasaponins in

feedstuffs [96] and commonly used food items [97] has been established. In addition to their safety, the quantification of soyasaponins in commonly used soy foods has been determined as well [98].

Chapter III. Materials and Methods

Soyasaponin I, III, and Soyasapogenol B Preparation

Soyasaponins I and III were isolated from soybeans that were grown as part of a study evaluating elicitor spray on soybeans and soyasaponin content at the University of Arkansas, as well as from a mixture of soyasaponins that was generously provided by Organic Technologies (Coshocton, OH). Total group B soyasaponins were extracted from whole soybeans by first coarsely grinding with a spice grinder to break the seeds, and then finely ground using a cyclone mill (UDY Cyclone Lab Sample Mill Model 3010-030), after which the ground soybean meal was passed through a 60 mesh sieve. The ground soybean meal was defatted using *n*-hexane in a Soxhlet apparatus. The remaining defatted soybean meal was then extracted for 2.5 hours with 70% ethanol in an Erlenmeyer flask on an orbital shaker at room temperature. Afterwards, the extraction mixture was filtered through Whatman no. 1 filter paper and collected in a round bottom flask. The soybean extract was rotary evaporated at 30°C. The remaining syrup-like residue was resuspended in water and freeze dried. HPLC analysis of the resulting soybean extract showed 10 different soyasaponins and there were no interfering isoflavones present. The soybean extract was fractionated via semi-prep HPLC as well as used as the starting material for alkaline hydrolysis.

Alkaline hydrolysis was used to convert DDMP-conjugated soyasaponins into non-DDMP soyasaponins, which was necessary for acquiring soyasaponins I and III. A modified method from Zhang *et al.* [99] was used for the alkaline hydrolysis, beginning with 100 mg of crude soyasaponin extract which was suspended in anhydrous methanol containing 5% NaOH (v/v). The mixture was placed in a closed reaction vessel and placed in an 80°C water bath for 30 minutes. After cooling, the reaction mixture was neutralized with concentrated HCl, diluted

with D.I. water to 50% methanol, and loaded on a preconditioned 10g C18 Sep-Pak column (Grace Davison, Deerfield, IL). The column was washed with water (100 ml) and the hydrolyzed mixture was then eluted with 80% methanol (50ml) and collected to be dried. Finally, the column was rinsed with 100% methanol to remove any remaining non-polar compounds; the column could then be reused. After rotary evaporation and subsequent freeze drying, the hydrolysis powder was subjected to HPLC analysis for confirmation of degree of hydrolysis and soyasaponins present. Soyasaponins V, I, II, and III were obtained in this manner via fractionation by semi-prep HPLC (Shimadzu Prominence CBM-20 Communications Bus Module, SPD-M20A Diode Array Detector, LC-20AB Liquis Chromatograph Pump) utilizing a reverse phase C18 YMC-Pack ODS-AM 250 x 10mm I.D. column.

The harsh conditions provided by 3N HCl and a boiling water bath are sufficient to cleave all carbohydrate moieties from the triterpene unit, but leave the aglycone 5-ring structure intact, and thus soyasapogenol B will be produced by acid hydrolysis using a modified method from Hu *et al.* [74]. 250 mg of group B soyasaponins provided by Organic Technologies (Coshocton, OH) were refluxed with 100 ml of 3N HCl in a boiling water bath for 45 minutes. The reaction mixture was quickly cooled and neutralized with 10N NaOH. The neutralization process resulted in salt forming due to the interaction of HCL + NaOH $\rightarrow$ NaCl + H_2O. The reaction mixture was desalted and the remaining sugars were removed by loading the mixture on a preconditioned 10g C18 Sep-Pak column (Grace Davison, Deerfield, IL) and washed with increasing percentages of methanol. The soyasapogenol B fraction is eluted using 200ml of 100% methanol. The fraction was rotary evaporated at 30°C, suspended in water, and then transferred to another flask to be freeze dried. The resulting powder is then ready to be further purified via semi-prep HPLC (Shimadzu Prominence CBM-20 Communications Bus Module,

SPD-M20A Diode Array Detector, LC-20AB Liquis Chromatograph Pump) utilizing a reverse phase C18 YMC-Pack ODS-AM 250 x 10mm I.D. column.

Cell Culture

Caco-2 human colon cancer cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD) at passage number 18 and cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% Non-essential amino acids, 2% antibiotic-antimicotic, and incubated at 37°C in a humidified 5% CO_2 atmosphere. All media components and reagents were obtained from Gibco® through Life Technologies (Carlsbad, CA).

Cell Viability Assay

Cells at passage numbers 30-31 were used for the following proliferation assays. Cellular proliferation and viability were measured using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega Corp. Madison, WI). This assay is a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays. The CellTiter reagent contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES).

The MTS tetrazolium compound is bio-reduced by living cells into a colored formazan product which is soluble in culture medium. The assays are performed by adding a small amount of the CellTiter reagent directly to culture wells in a 96 well plate, incubating for 1-4 hours (depending on the metabolic rate of your cells), and then recording the absorbance at 490nm with

a plate reader (Synergy HT Multi-Mode Microplate Reader, BioTek Instruments, Inc., Winooski, VT). The quantity of formazan product measured by the absorbance at 490nm is directly proportional to the number of living cells in culture, as seen in **Figure 3.**

To perform this assay, (2×10^3) Caco-2 cells in 100µl of DMEM containing 10% fetal bovine serum were seeded in the wells of a 96 well plate and incubated at 37°C and 5% CO_2. After stable attachment (48 hrs) media was removed and replaced with test media containing soyasaponins I, III, and sapogenol B at concentrations of 0, 300, 600, and 900 ppm.

Viability measurements were made at 4 different time points: 0, 24, 48, and 72 hrs. this was accomplished by adding 20µl of CellTiter reagent directly to the wells containing cells as well as wells containing only the test media (to be used as a sample control) and incubating for 4 hours before measuring the absorbance at 490nm. After all absorbance readings had been made, corrections were made for the background absorbance of the control media as well as the sample control media which contained soyasaponins. Absorbances were then converted into cell numbers using an equation from the Caco-2 cell standard curve completed earlier in which a serial dilution of cells was prepared in triplicate in a 96 well plate which ranged from 350 cells per ml to 70,000 cells per ml. After the dilution was complete, CellTiter reagent was added and absorbances were recorded after 4 hours. After absorbances were converted into cell numbers, cell growth curves were then plotted.

Light Microscopy of Cells

Cell morphology was observed using a Nikon Eclipse E400 light microscope with Nikon Camera Head DS-Fi1. Cells from passage number 41 were used to visualize morphology changes. After 72 hours of treatment with soyasaponin I, III, or soyasapogenol B, cells were

harvested and an aliquot was taken from the cell suspension. A portion of the aliquot was then stained with trypan blue dye to determine viable from non-viable cells and also to provide contrast. 10µl of the aliquot was then pipetted onto a slide and covered with a cover-slip. The prepared slides were viewed under low power magnification until an acceptable cell was identified. The cell of interest was then viewed under higher power (400x). Using the Nikon camera software, contrast and color was adjusted to provide the best possible image, after which, a still shot was taken and saved for later viewing.

PKC Assay

Cells at passage numbers 39-40 were used for PKC enzyme activity assays. PKC activity was determined with the PepTag® Non-Radioactive PKC assay kit (Promega Corp. Madison, WI). The PepTag® Assay utilizes a brightly colored fluorescent peptide that it highly specific for PKC, which phosphorylates the PKC peptide, thus changing its net charge from +1 to -1. The change in net charge of the substrate allows the phosphorylated and non-phosphorylated versions of the substrate to be rapidly separated on an agarose gel. The color of the PepTag® Peptide substrate is imparted by the addition of a dye molecule, which allows for easy visualization of the bands during the electrophoresis process. After electrophoresis, the bands can be excised, re-solubilized, and quantified by reading their absorption on a plate reader at 570nm.

For this assay, 5×10^6 Caco-2 cells were seeded in a $75cm^2$ flask. After stable attachment, media was removed and replaced with test media containing 300, 600, and 900ppm of soyasaponins I and III and 50, 100, and 150ppm of sapogenol B, as well as media containing no soyasaponins or soyasapogenol B. All media except that of the negative control contained

12-*O*-tetradecanoyl phorbol 13 acetate (TPA) (100ng/ml), a known PKC stimulator. The cells were then incubated for 72 hrs at 37°C and 5% CO_2.

After the incubation time was over, all media was collected, cells were rinsed with phosphate-buffered saline (PBS), the cells were trypsinized, removed from the flask, and the flask was rinsed again with PBS to collect all remaining cells. To pelletize the cells, the mixture was centrifuged and all supernatant was completely removed. The cells were then suspended in 0.5ml of cold PKC extraction buffer (**Table 2**) and then homogenized by using multiple freeze thaw cycles and a probe sonicator (XL-2000 Q Sonica, LLC, Newton, CT). The cell lysate was then centrifuged for 5 minutes at 4°C, 14,000 x g in a microcentrifuge. The supernatant was passed over a 1ml column of Whatman diethylaminoethyl cellulose that had already been preconditioned with PKC extraction buffer. The column was washed with 5ml of PKC extraction buffer. The PKC-containing fraction was eluted using 5 ml of PKC extraction buffer containing 200mM NaCl.

For each sample, the PepTag® Reaction 5X buffer, PepTag® C1 peptide, PKC Activator 5X solution, and DI water was added to a 0.5ml microcentrifuge tube and kept on ice until the sample was ready to be added. At time zero, the tubes were removed from the ice and incubated in a 30°C water bath for 2 minutes. Then, the sample was added and incubated for another 30 minutes at 30°C. The reaction was stopped by placing the tubes in a boiling water bath for 10 minutes. The samples were stored at 4°C in the dark until they were ready to be loaded on the gel.

A 0.8% agarose gel was prepared using 50mM Tris-HCl pH 8.0. The solution was heated until the agarose was completely dissolved and was allowed to cool to approximately 60°C before pouring into the gel tray. Only one comb was used and it was placed in the middle of the

gel. The gel was allowed to solidify for 30 minutes. The horizontal gel apparatus was assembled according to the manufacturer's specifications.

After the gel had solidified, it was placed in the electrophoresis chamber and the comb was gently removed. 50mM Tris-HCl (pH 8.0) was poured over the gel until the gel was covered completely. Before loading the samples, 1µl of 80% glycerol was added to each sample to ensure it stayed in the well. Immediately after the last sample was loaded, the gel was run at 100V for 15-18 minutes. Once the electrophoresis was complete, the gel was removed from the chamber and photographed under UV light to provide for greater sensitivity.

Using a scalpel, the negatively charged bands were quickly excised to avoid diffusion. The excised band was then placed into a 1.5ml microcentrifuge tube and heated at 95°C until the gel slice was melted. 125µl of the hot agarose was pipetted into a tube containing 75µl of Gel Solubilization Solution and 50µl of glacial acetic acid. The mixture was quickly vortexed and transferred to a well in a 96-well plate. After all the samples had been transferred to the 96-well plate, the absorbance was read at 570nm. The plate reader was blanked by using liquefied agarose without PapTag® Peptide. The activity of PKC in the samples was then calculated using the absorbances seen in the PKC positive control dilution assays.

Statistical Analysis

Data will be analyzed using Statistical Analysis System (Version 9.2; SAS Institute Inc., Cary, NC). Results will be analyzed using one-way analysis of variance using an alpha level of 0.05 followed by Fisher's least significant difference (LSD) test. Data is presented as means ± standard error of the mean (SEM).

Chapter IV. Results

Soyasaponins I, III, and Soyasapogenol B inhibit proliferation of Caco-2 Cells.

Treatment of cultures of Caco-2 cells with soyasaponins I, III and soyasapogenol B decreased cell proliferation in a time and concentration-dependent manner (**Table 3, Figure 4, & 5**). As early as 24 h after initiation of soyasapogenol B treatment, viable cell numbers were significantly reduced by 14.7-62.4%, compared with the control, at concentrations of 100-150 ppm respectively ($p<0.05$) (**Table 3**). After 48 h of treatment, Soyasaponins I and III at all concentrations reduced viable cell numbers by 9.6-34.6% ($p<0.05$) (**Table 3**). Similarly, after 72 h of soyasaponins I, III, and soyasapogenol B treatment viable cell numbers were significantly reduced by 46.3-94.3%, compared with the control ($p<0.05$) (**Table 3**).

Light Microscopy

Figure 6 shows the light microscope observations of Caco-2 cells treated with 0, 300, 600, and 900 ppm of soyasaponins I and III (**Figure 6. (b) & (c)**) as well as 50, 100, and 150 ppm of soyasapogenol B (**Figure 6. (d)**). When the dose of soyasaponins increased, the surface of Caco-2 cells became rougher, and the cell shape changed from round to irregular. As the dose of soyasaponin I and III reached 900 ppm, breaks were seen on the surface of Caco-2 cells. At the highest concentrations of soyasapogenol B, complete deformation of Caco-2 cells was observed (**Figure 6 (d)**).

PKC Assay

Expression of the proliferation enzyme PKC was decreased in cells treated with soyasaponins I, III, and soyasapogenol B (**Table 4)**. Soyasaponin I reduced PKC expression in a

dose-dependent manner, ranging from 9.0-27.3% reduction in PKC activity compared to the control ($p<0.05$). Soyasaponin III also reduced PKC activity in a dose-dependent manner. Reductions of 18.2-40.0% were seen in the soyasaponin III group ($p<0.05$) . The soyasapogenol B group showed reductions in PKC ranging between 56.4-70.9%. The 100 and 150 ppm groups showed significantly greater reductions than the 50 ppm group ($p<0.05$), but 150 ppm was not significantly greater than the 100 ppm group.

Chapter V. Discussion

Numerous epidemiological and experimental studies have suggested that soy contains several bioactive phytochemicals. However, the possible mechanisms of their effects are not fully understood. Confusion from different animal models, cell lines, and test materials have further complicated the task of identifying what soy phytochemicals are responsible for the health effects linked with soy consumption.

In the present study, the effects of the predominant soyasaponin in heat treated soy products (soyasaponin I) as well as its two primary metabolites (soyasaponin III and soyasapogenol B) were tested to evaluate their effect on cell growth, cell morphology changes, and proliferation-related enzyme activity of Caco-2 human colon cancer cells. Soyasaponins I, III, and soyasapogenol B effectively inhibited the growth rate and survival of human colon cancer cells, altered cell morphology, and inhibited the TPA-stimulated PKC activity in a dose-dependent manner.

Soyasaponins I, III and soyasapogenol B decreased cell proliferation in human colon cancer cells in a time and concentration-dependent manner (**Table 3**). Cell proliferation and viability data from this study compare favorably with previous research [65-68, 72]. However, these studies utilized different cell lines HT-29 and HCT-15, different soyasaponin mixtures, and concentrations. When comparing the data of these studies, it is important to look at the growth curves of both the control cells and the treatment cells. Control cells display an exponential growth curve, but depending on the cell line, the slope of that line may vary in steepness. The doubling time of HT-29 cells and HCT-15 cells vary, but is approximately 24 hrs. Caco-2 cells on the other hand are metabolically slower and double approximately every 62 hours. Depending on the study, cell proliferation and viability studies used different methods. Some

studies plated a specific density of cells in 35mm cell culture plates and determined cell count and viability using a hemocytometer and trypan blue [64-67], while others used 96 well plates and a chemical reagent which would be bio-reduced to form a colored product which could be quantified using a plate reader [63,73]. Either method produces similar growth curve patterns. So, even while all the control growth curves are not identical, they follow an exponential pattern. Treatment-cell growth curves vary from study to study, but they too follow a similar pattern, in that they always respond in a dose-dependent manner and are generally similar to the negative control curve, but differ in steepness. An example of this can be seen in **figure 7** [66]. In the present study, the cell growth curves of the treatment groups, up to 48 hours, resemble those of the before mentioned research [66]. Where this study varies from previous research is that depending on the treatment group, the growth curves do not continue upwards after 48 hours. Depending on treatment group, the cells plateau after 24 hours or begin to die off after 48 hours eventually returning to near 0 hour cell numbers after 72 hours. The soyasapogenol B data tells a somewhat different story because of its increased bioactivity. Soyasapogenol B at 50 and 100 ppm begin to follow the growth curves of previous research up to 24 hours, but after which cell proliferation stops and cell viability drops off dramatically leading up to the 48 and 72 hour time points.

Comparison with other studies utilizing mixtures of soyasaponins shows that the purified soyasaponins used in this study were more effective in reducing cell growth at lower concentrations. Tsai *et al.* [63] reduced cell growth by 27% at 72 hours when treated with 600 ppm of soyasaponins compared with control cells. However, the 300 ppm soyasaponin group was not significantly different from the control cells. In the current study, soyasaponins I and III at 300 and 600 ppm reduced cell growth by 46-55% at 72 hours (**Table 3**). Three other studies

[64, 66, 67] which utilized a mixture of soyasaponins demonstrated that cell growth could be significantly reduced by 150, 300, and 600 ppm of soyasaponins after 48 and 72 hours, but the percent reduction in cell growth compared to the control cells was not listed. Even though the percent reduction in cell growth was not listed in these studies, it is clear when looking at the cell growth curves that these soyasaponin mixtures did not have the same affect that the purified soyasaponins in this study did. When reaching the 48 and 72 hour time points in the previously listed studies, cell growth curves were still progressing steadily upwards, but in the current study, cell growth curves were already plateauing (48 hours) or decreasing (72 hours) (**Figure 4. a & b**).

In the only other study that utilized individual soyasaponins [73], soyasaponin I, III and soyasapogenol B were utilized at 6, 12, 25, and 50 ppm. The study also utilized a group B soyasaponin mixture at those same concentrations. After 72 hours of treatment, the group B mixture and soyasaponin I groups had no effect on cell growth. Soyasaponin III at its highest concentration reduced cell growth by approximately 25%, it's difficult to make a comparison to this study, but soyasaponin III at 300 ppm reduced cell growth by 49% (**Table 3**). This is a higher percentage, but also a higher concentration. Soyasapogenol B at 50 ppm reduced cell growth by approximately 90-95% in the study mentioned above. The current study reduced cell growth by 87% (**Table 3**), which is similar in comparison to the 90-95% reduction seen in the other study. Gurfinkel *et al.* [73] demonstrated that the soyasaponin mixture and soyasaponin I did not have an effect, but soyasaponin III (with fewer sugars) and soyasapogenol B (no sugars) decreased cell growth. Evidence from Gurfinkel *et al.* [73] and the current study indicate that differences in soyasaponin lipophilicity have a significant effect on bioactivity and resulting

cancer cell growth. All this lends to the hypothesis that with greater lipophilicity there is also greater bioactivity.

By viewing under a microscope, it became evident that when treated with increasing concentrations of soyasaponins and soyasapogenol B, cell membranes became more rough and irregular. At the highest concentrations of soyasaponin and soyasapogenol B complete deformation of Caco-2 cells was observed (**Figure 6**). In the study by Tsai *et al*, [63] as concentrations of soyasaponins increased, the surface of the HT-29 cells became more rough and irregular leading up to 600 ppm. At 1200 ppm, breaks were seen on the surface of the cells, and at 2400 ppm, complete deformation of the HT-29 cells was observed. The changes in cell morphology are suggestive that soyasaponins may be binding to or inserting into the cell membrane. This is particularly relevant because the membranes of some cancer cells contain more cholesterol than normal cell membranes. Because of this, it is also likely that soyasaponins bind more to cancer cells, and thus in the intestine, soyasaponins can bind to the mucosal cell membrane, change its physiology, and as a result reduce cancerous cell growth. A relationship between cell morphology and increased lipophilicity of soyasaponins and soyasapogenol B was observed in this study. Morphological changes observed through microscopy of cells indicate that as lipophilicity increases, cell morphology and cell membrane integrity become more compromised. Cell membranes become rough and irregular with treatment of soyasaponins I and III. With treatment of soyasapogenol B, cell membranes were irregular and eventually deformed to the point where the membranes are no longer intact and the cell was completely deformed.

PKC is one of the markers for cell proliferation and its activity increases as the cells undergo the proliferation process. As shown in this study, the addition of soyasaponins I, III,

and soyasapogenol B to the cell culture media effectively reduced the expression of PKC in colon cancer cells. Previous research [64-66] has shown similar results when using crude mixtures of group B soyasaponins and HT-29 colon cancer cells. Kim *et al.*, [65] demonstrated that 600 ppm soyasaponins significantly reduced PKC expression after 72 hours. Treatment with 150 and 300 ppm did not significantly reduce PKC expression. In the study by Oh *et al.*, [66] PKC expression was significantly reduced by 300 and 600 ppm soyasaponin treatment. However, there was no dose dependent effect. The reduction in PKC activity in the 600 ppm group was not significantly greater than the 300 ppm group, 21.4 and 20% respectively.

In this study, there was a dose dependent effect seen in the soyasaponin I group. For the 300, 600, and 900 ppm groups, PKC activity was reduced by 9.1, 16.4, and 27.3% respectively (**Table 4**). Soyasaponin III followed a similar pattern, and reductions for this group are as follows: 18.2, 32.7, and 40.0%. Depending on the soyasaponin, the percent reductions in PKC activity of this study are close to those of the previously mentioned studies [63-65] which used soyasaponin mixtures. Soyasaponin I reduction was less than the reported values in these studies when used at the same concentrations, but soyasaponin III had a greater reduction at the 600 ppm concentration compared to mixtures of soyasaponins at that same concentration.

Initially, it appeared that the soyasapogenol B group had reduced the PKC activity of the cells by 56.4, 65.5, and 70.9% (**Table 4**). However, because of its increased bioactivity, after 72 hours cell viability was extremely low, therefore, the measured reduction in PKC activity may not have been due to reduced expression of the protein by the cells, but rather because the cells were no longer alive to produce the protein.

Previous report has indicated that soyasaponins possess surface-active properties [67]. Transmission electron microscopy imaging indicates that soyasaponins induced the formation of

vacuoles and deformations in plasma and nuclear membranes of cells [72]. By binding to or inserting themselves into the cell membrane, they are able to disrupt normal cell function, which appears to be the case with PKC. Previous studies have suggested that the disruption by the soyasaponins somewhat modulates the translocation of PKC from the cytosol to the cell membrane [65, 66], thus reducing its expression. As mentioned earlier, soyasaponin-cell membrane interactions appear to be the key mechanism by which soyasaponins elicit their effects. Proliferation and viability data, microscopy results, and PKC activity from this study all indicate that the bioactivity of soyasaponins and soyasapogenol B are directly related to their molecular structure and more importantly their lipophilicity. The differences in bioactivity are likely due to the fact that with increases in lipophilicity, there is more soyasaponin/ soyasapogenol B interaction with the cancer cell membranes, which are rich in phospholipids and cholesterol.

Chapter VI. Conclusion

The predominant soyasaponin in heat treated soy products, soyasaponin I, is metabolized first into soyasaponin III and finally into soyasapogenol B by colonic microflora. In summary, cell proliferation and PKC activity were reduced in a dose dependent manner when treated with these phytochemicals. Data from proliferation and PKC assays as well as morphological analysis indicates that the suppression of cell growth and disruption of cell membrane integrity is intensified with increases in lipophilicity of the soyasaponins and soyasapogenol B, meaning that soyasapogenol B is greater than soyasaponin III which is greater than soyasaponin I. The results of this study indicate that the predominant soyasaponin in heat treated soy products and its two metabolites, at physiologically relevant doses, can suppress colon cancer growth. These findings suggest that purified soyasaponins and soyasapogenol B may be a colon cancer suppressive component of soy which warrants further examination as a potential nutraceutical or functional food.

Appendix

Figure 3. CellTiter 96® AQ_{ueous} One Solution Caco-2 Cell Standard Curve

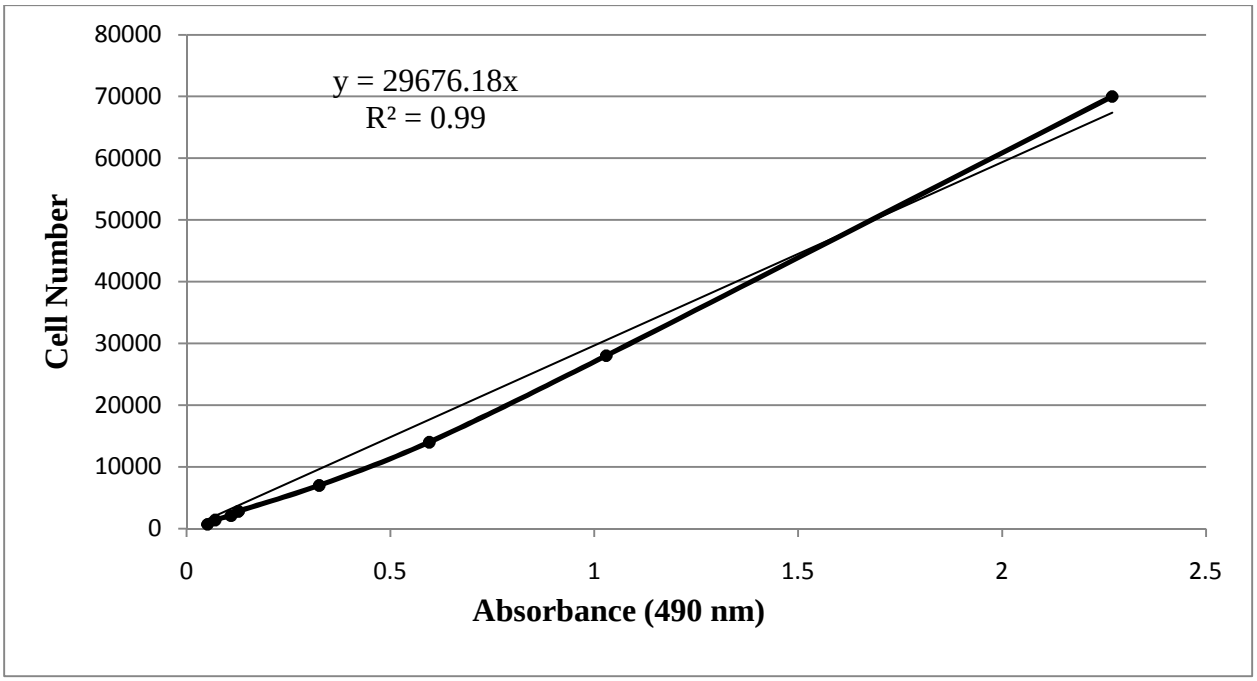
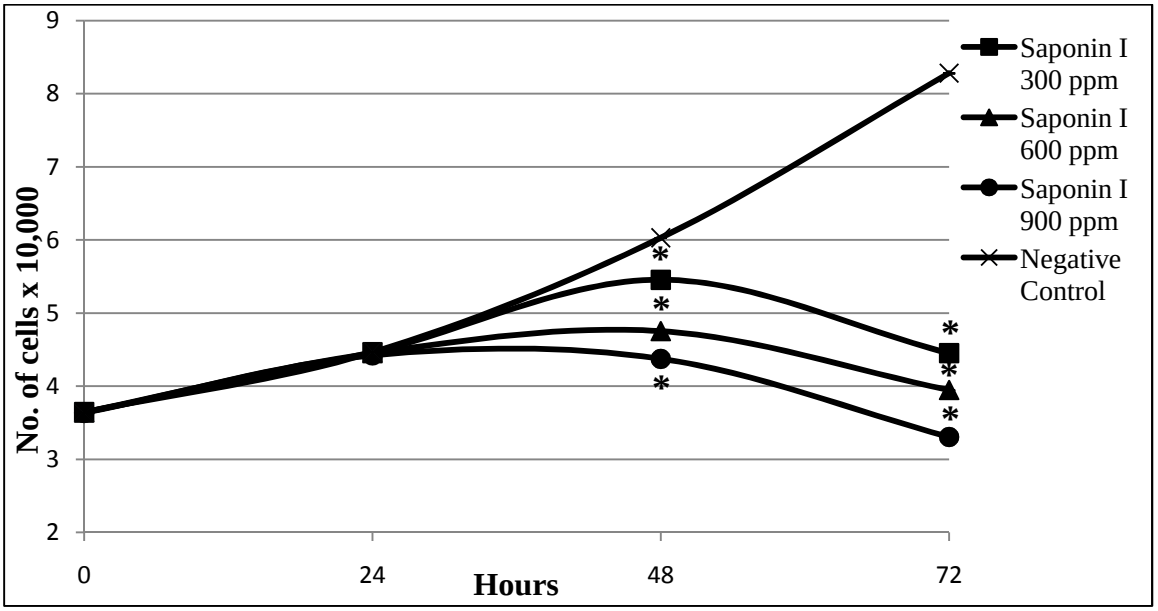


Table 2. PKC Extraction Buffer

Ingredients	Final Concentration
Tris-HCL (pH 7.4)	25mM
EDTA	0.5mM
EGTA	0.5mM
Triton® X-100	0.05%
β-mercaptoethanol	10mM
Leupeptin	1µg/ml
Aprotinin	1µg/ml

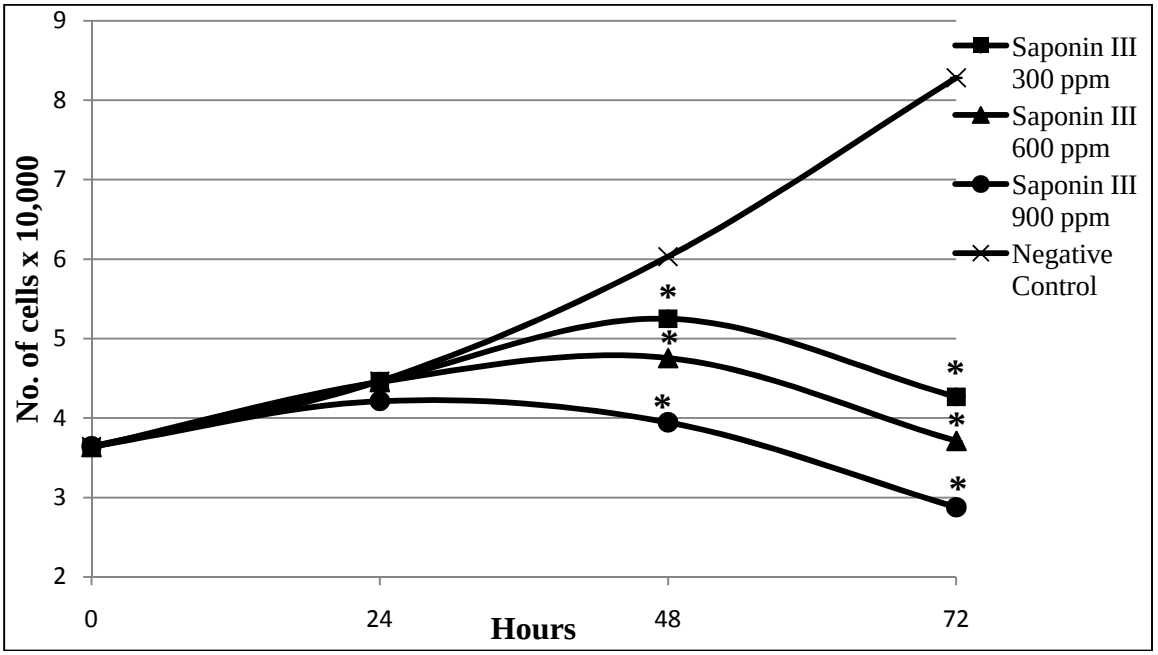
0.5ml Stock solution (100mM PMSF in 100% EtOH) per 100ml of PKC extraction buffer added just before use.

Figure 4. (a) Soyasaponin I effects on CaCo-2 cell proliferation



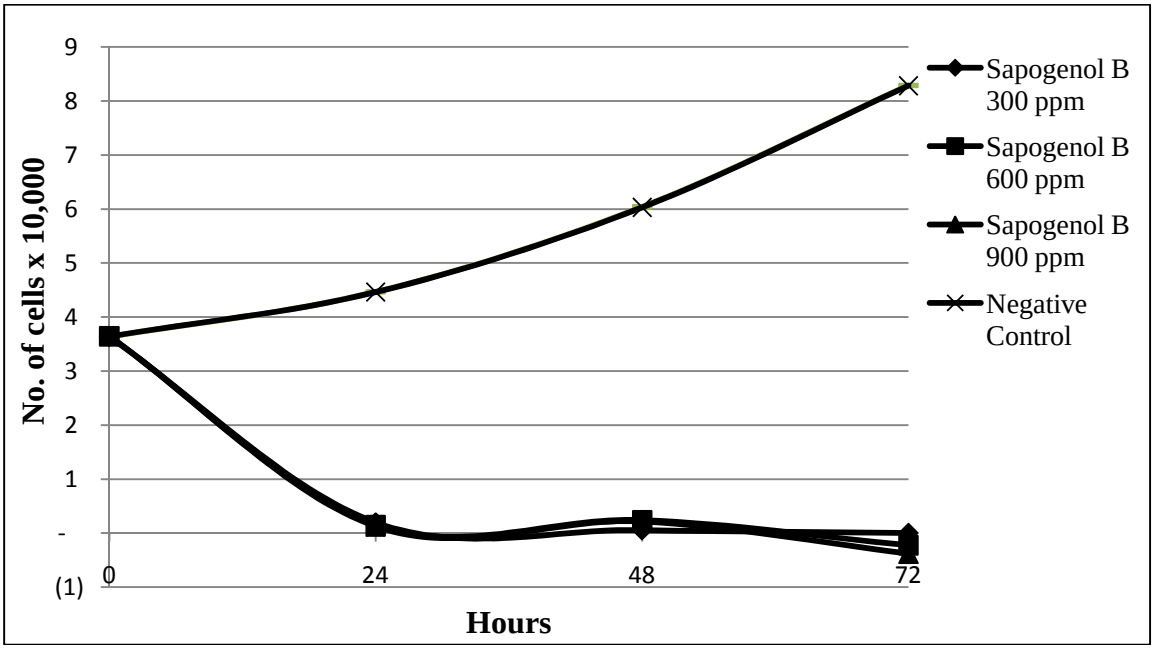
Different concentrations of Soyasaponin I at each incubation time were compared against the negative control using Dunnett's test. Points marked with (*) represent significant differences at the P<0.05 level.

(b) Soyasaponin III effects on Caco-2 cell proliferation

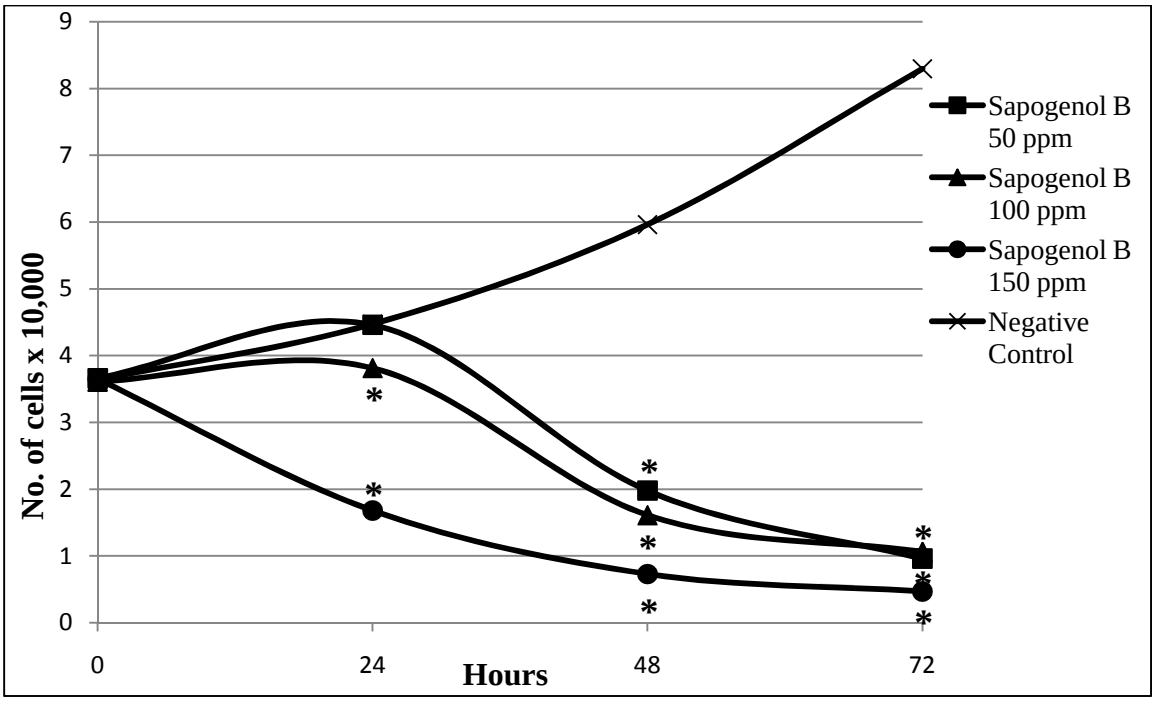


Different concentrations of Soyasaponin III at each incubation time were compared against the negative control using Dunnett's test. Points marked with (*) represent significant differences ($p < 0.05$)

Figure 5. (a) Soyasapogenol B effects on Caco-2 cell proliferation



(b) Soyasapogenol B effects on Caco-2 cell proliferation



Different concentrations of Soyasapogenol B at each incubation time were compared against the Negative Control using Dunnett's test. Points marked with (*) represent significant differences ($p < 0.05$)

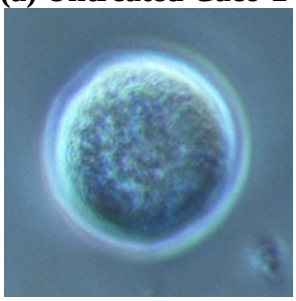
Table 3.
Soyasaponin I, III, and soyasapogenol B Percent Reduction in Cell Viability

Treatment	Conc. (ppm)	24 Hours		48 Hours		72 hours	
		Percent Reduction	SEM	Percent Reduction	SEM	Percent Reduction	SEM
Soyasaponin I	300	0.1	0.23	9.6[a]	1.45	46.3[a]	1.80
	600	0.5	0.23	21.2[b]	0.95	52.3[b]	2.47
	900	1.1	0.40	27.5[c]	0.78	60.1[d]	0.14
Soyasaponin III	300	0.5	0.23	13.0[a]	0.41	48.5[a]	0.28
	600	5.8[a]	0.15	21.2[b]	1.00	55.2[c]	0.15
	900	5.8[a]	0.31	34.6[d]	1.57	65.3[e]	1.54
Soyasapogenol B	50	0.3	0.12	67.2[e]	1.14	87.2[f]	0.75
	100	14.7[b]	6.87	73.3[f]	1.90	88.4[f]	0.32
	150	62.4[c]	0.99	87.9[g]	0.43	94.3[g]	0.29

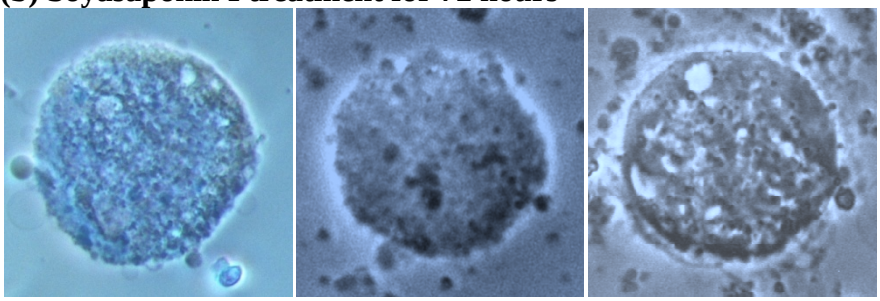
Data represents the percent reduction (%) compared with negative control and the standard error of the mean (SEM) (n = 3). Values in a column without common superscripts are significantly different ($p < 0.05$).

Figure 6. Morphological effects of treatment with different concentrations of Soyasaponin I, III, and soyasapogenol B.

(a) Untreated Caco-2 cell

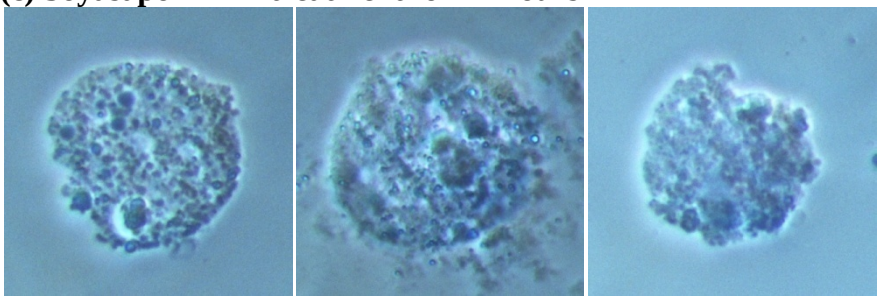


(b) Soyasaponin I treatment for 72 hours



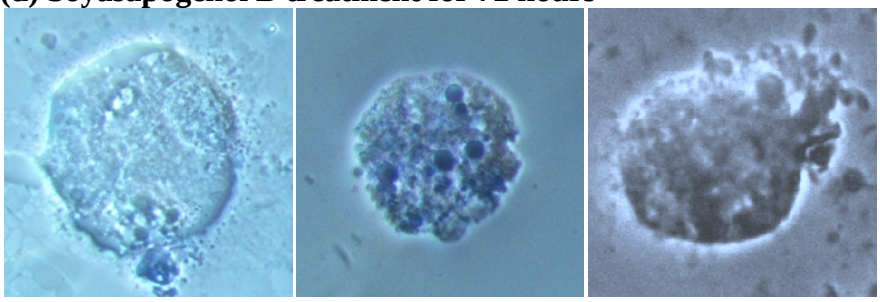
(i) 300ppm (ii) 600 ppm (iii) 900 ppm

(c) Soyasaponin III treatment for 72 hours



(i) 300ppm (ii) 600 ppm (iii) 900 ppm

(d) Soyasapogenol B treatment for 72 hours



(i) 50 ppm (ii) 100 ppm (iii) 150 ppm

Figure 7. Example of typical growth curves in a proliferation assay. Figure taken from Oh *et.al.*, 2001 [66]

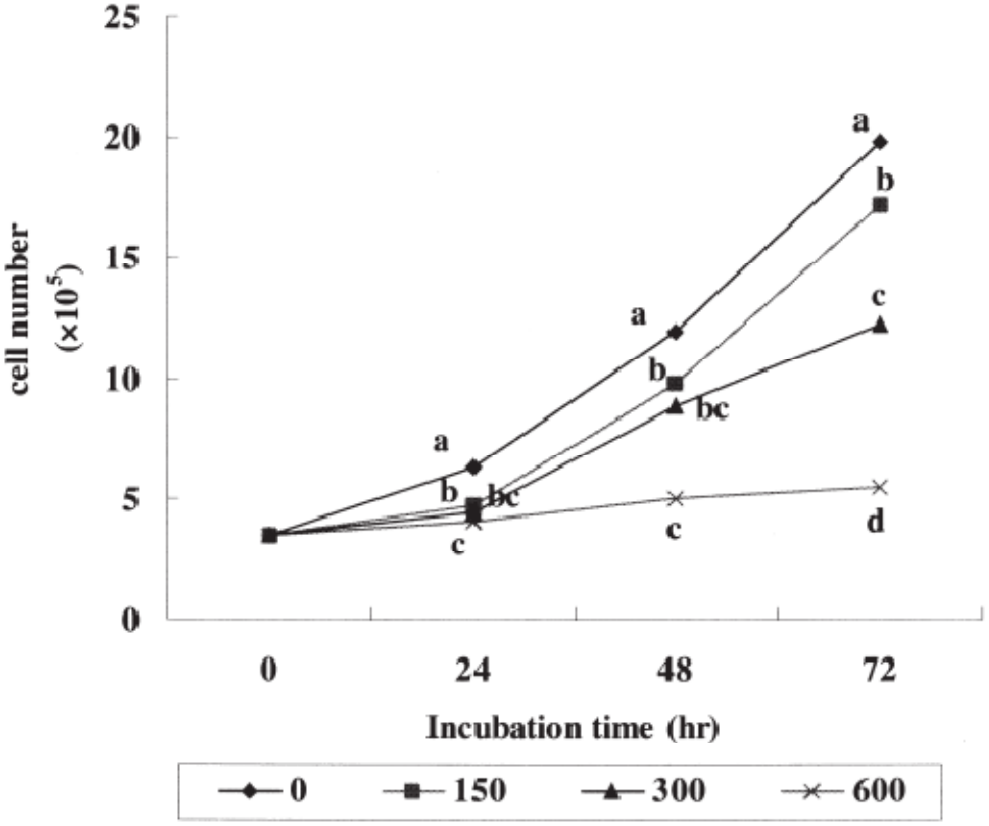


Table 4. Reduction of PKC by soyasaponins I, III, and sapogenol B

Treatment	Conc. (ppm)	Percent Reduction (%)	SEM
Soyasaponin I	300	9.1[a]	1.82
	600	16.4[b]	1.82
	900	27.3[c]	1.82
Soyasaponin III	300	18.2[b]	3.15
	600	32.7[c]	1.82
	900	40.0[d]	3.15
Soyasapogenol B	50	56.4[e]	3.15
	100	65.5[f]	1.82
	150	70.9[f]	1.82

Data represents the percent reduction (%) compared with control (cells+100ng/ml TPA) and the standard error of the mean (SEM) (n = 3). Values in a column without common superscripts are significantly different ($p < 0.05$).

Literature Cited

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ: **Cancer statistics, 2009**. *CA: a cancer journal for clinicians* 2009, **59**(4):225.

2. Lynch HT, de la Chapelle A: **Hereditary colorectal cancer**. *The New England journal of medicine* 2003, **348**(10):919.

3. Parkin DM: **International variation**. *Oncogene* 2004, **23**(38):6329-6340.

4. Wiseman M: **The second World Cancer Research Fund/American Institute for Cancer Research expert report. Food, nutrition, physical activity, and the prevention of cancer: a global perspective**. *Proceedings of the Nutrition Society* 2008, **67**(03):253-256.

5. Fearon ER, Vogelstein B: **A genetic model for colorectal tumorigenesis**. *Cell* 1990, **61**(5):759-767.

6. Kinzler KW, Vogelstein B: **Lessons from hereditary colorectal cancer**. *Cell* 1996, **87**(2):159-170.

7. Arnold CN, Goel A, Blum HE, RichardBoland C: **Molecular pathogenesis of colorectal cancer**. *Cancer* 2005, **104**(10):2035-2047.

8. Easwaran V, Song V, Polakis P, Byers S: **The ubiquitin-proteasome pathway and serine kinase activity modulate adenomatous polyposis coli protein-mediated regulation of beta-catenin-lymphocyte enhancer-binding factor signaling**. *Journal of Biological Chemistry* 1999, **274**(23):16641.

9. Luchtenborg M, Weijenberg MP, Roemen GMJM, de Bruine AP, van den Brandt PA, Lentjes MHFM, Brink M, van Engeland M, Goldbohm RA, de Goeij AFPM: **APC mutations in sporadic colorectal carcinomas from The Netherlands Cohort Study**. *Carcinogenesis* 2004, **25**(7):1219.

10. Ilyas M, Tomlinson IPM: **The interactions of APC, E-cadherin and β-catenin in tumour development and progression**. *The Journal of pathology* 1997, **182**(2):128-137.

11. Ilyas M, Tomlinson IPM, Rowan A, Pignatelli M, Bodmer WF: **β-Catenin mutations in cell lines established from human colorectal cancers**. *Proceedings of the National Academy of Sciences of the United States of America* 1997, **94**(19):10330.

12. Baker SJ, Fearon ER, Nigro JM, Hamilton SR, Preisinger AC, Jessup JM, VanTuinen P, Ledbetter DH, Barker DF, Nakamura Y: **Chromosome 17 Deletions an p 53 Gene Mutations in Colorectal Carcinomas**. *Science(Washington)* 1989, **244**(4901):217-217.

13. Yamaguchi A, Makimoto K, Goi T, Takeuchi K, Maehara M, Isobe Y, Nakagawara G: **Overexpression of p53 protein and proliferative activity in colorectal adenoma**. *Oncology* 1994, **51**(3):224-227.

14. Yamaguchi M, Hayashi Y, Matsuoka S, Takahashi T, Matsukage A: **Differential effect of p53 on the promoters of mouse DNA polymerase β gene and proliferating-cell-nuclear-antigen gene**. *European Journal of Biochemistry* 1994, **221**(1):227-237.

15. Bird RP: **Role of aberrant crypt foci in understanding the pathogenesis of colon cancer**. *Cancer letters* 1995, **93**(1):55-71.

16. Lipkin M: **Biomarkers of increased susceptibility to gastrointestinal cancer: new application to studies of cancer prevention in human subjects**. *Cancer research* 1988, **48**(2):235.

17. Lipkin M, Reddy B, Newmark H, Lamprecht SA: **Dietary Factors In Human Colorectal Cancer**. *Annual Review of Nutrition* 1999, **19**(1):545-586.

18. Bird RP, Good CK: **The significance of aberrant crypt foci in understanding the pathogenesis of colon cancer**. *Toxicology letters* 2000, **112**(Journal Article):395-402.

19. Caderni G, Giannini A, Lancioni L, Luceri C, Biggeri A, Dolara P: **Characterisation of aberrant crypt foci in carcinogen-treated rats: association with intestinal carcinogenesis**. *British journal of cancer* 1995, **71**(4):763.

20. McLellan EA, Medline A, Bird RP: **Sequential analyses of the growth and morphological characteristics of aberrant crypt foci: putative preneoplastic lesions**. *Cancer research* 1991, **51**(19):5270.

21. Pretlow TP: **Aberrant crypt foci and K-ras mutations: earliest recognized players or innocent bystanders in colon carcinogenesis?** *Gastroenterology* 1995, **108**(2):600-603.

22. Pretlow TP, Barrow BJ, Ashton WS, O'Riordan MA, Pretlow TG, Jurcisek JA, Stellato TA: **Aberrant crypts: putative preneoplastic foci in human colonic mucosa**. *Cancer research* 1991, **51**(5):1564.

23. Roncucci L, Medline A, Bruce WR: **Classification of aberrant crypt foci and microadenomas in human colon**. *Cancer Epidemiology Biomarkers & Prevention* 1991, **1**(1):57.

24. Roncucci L, Modica S, Pedroni M, Tamassia MG, Ghidoni M, Losi L, Fante R, Di Gregorio C, Manenti A, Gafa L: **Aberrant crypt foci in patients with colorectal cancer**. *British journal of cancer* 1998, **77**(12):2343.

25. Roncucci L, Stamp D, Medline A, Cullen JB, Robert Bruce W: **Identification and quantification of aberrant crypt foci and microadenomas in the human colon**. *Human pathology* 1991, **22**(3):287-294.

26. Bird RP: **Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: preliminary findings**. *Cancer letters* 1987, **37**(2):147-151.

27. Schottenfeld D, Beebe-Dimmer J: **Advances In Cancer Epidemiology: Understanding Causal Mechanisms and the Evidence for Implementing Interventions**. *Annual Review of Public Health* 2005, **26**(1):37-60.

28. Yeole BB, Kurkure AP, Koyande SS: **Geographic variation in cancer incidence and its patterns in urban Maharashtra, 2001**. *Asian Pacific Journal of Cancer Prevention* 2006, **7**(3):385.

29. Kolonel LN, Hinds MW, Hankin JH: **Genetic and environmental factors in experimental and human cancer**. *Japan Scientific Societies Press: Tokyo, Japan* 1980(Journal Article):327–340.

30. McMichael AJ, McCall MG, Hartshore JM, Woodings TL: **Patterns of gastro-intestinal cancer in European migrants to Australia: the role of dietary change**. *International Journal of Cancer* 1980, **25**(4):431-437.

31. Parkin DM, Whelan SL, Ferlay J, Teppo L, Thomas DB: **Cancer Incidence in Five Continents Vol VIII. IARC Scientific publication No 155**. *Lyon: International Agency for Research on Cancer* 2002(Journal Article).

32. Perera FP: **Environment and cancer: who are susceptible?** *Science* 1997, **278**(5340):1068.

33. Potter JD: **Colorectal cancer: molecules and populations**. *JNCI Journal of the National Cancer Institute* 1999, **91**(11):916.

34. Birt DF, Hendrich S, Wang W: **Dietary agents in cancer prevention: flavonoids and isoflavonoids**. *Pharmacology & therapeutics* 2001, **90**(2-3):157-177.

35. Messina M, Barnes S: **The role of soy products in reducing risk of cancer**. *JNCI Journal of the National Cancer Institute* 1991, **83**(8):541.

36. Messina MJ, Persky V, Setchell KDR, Barnes S: **Soy intake and cancer risk: a review of the in vitro and in vivo data**. *Nutrition and cancer* 1994, **21**(2):113-131.

37. Akhter M, Inoue M, Kurahashi N, Iwasaki M, Sasazuki S, Tsugane S: **Dietary Soy and Isoflavone Intake and Risk of Colorectal Cancer in the Japan Public Health Center–Based Prospective Study**. *Cancer Epidemiology Biomarkers & Prevention* 2008, **17**(8):2128.

38. Messina M, Nagata C, Wu AH: **Estimated Asian adult soy protein and isoflavone intakes**. *Nutrition and cancer* 2006, **55**(1):1-12.

39. Nagata C, Nakamura K, Oba S, Hayashi M, Takeda N, Yasuda K: **Association of intakes of fat, dietary fibre, soya isoflavones and alcohol with uterine fibroids in Japanese women**. *British Journal of Nutrition* 2008, **101**(10):1427-1431.

40. Takata Y, Maskarinec G, Franke A, Nagata C, Shimizu H: **A comparison of dietary habits among women in Japan and Hawaii**. *Public health nutrition* 2007, **7**(02):319-326.

41. Shi J, Arunasalam K, Yeung D, Kakuda Y, Mittal G, Jiang Y: **Saponins from edible legumes: Chemistry, processing, and health benefits**. *Journal of medicinal food* 2004, **7**(1):67-78.

42. Oleszek W, Marston A: **Saponins in food, feedstuffs and medicinal plants**: Springer Netherlands; 2000.

43. Waller GR, Hostettman K, Marston A: **Saponins: Chemistry and Pharmacology of Natural Products**. *Journal of the American Chemical Society* 1996, **118**(35):8509.

44. Gestetner B: **A Rapid Paper Chromatographic Method for Separation and Identification of Soybean Sapogenols**. *Journal of chromatography* 1964, **13**(Journal Article):259-261.

45. Ireland PA, Dziedzic SZ: **Effect of hydrolysis on sapogenin release in soy**. *Journal of Agricultural and Food Chemistry* 1986, **34**(6):1037-1041.

46. Ireland PA, Dziedzic SZ: **Analysis of soybean sapogenins by high-performance liquid chromatography**. *Journal of Chromatography A* 1985, **325**(Journal Article):275-281.

47. Smith HM, Smith JM, Spring FS: **The triterpenoids--LVI:: The chemistry of the soyasapogenols A, B and C**. *Tetrahedron* 1958, **4**(1-2):111-131.

48. Kudou S, Tonomura M, Tsukamoto C, Uchida T, Sakabe T, Tamura N, Okubo K: **Isolation and structural elucidation of DDMP-conjugated soyasaponins as genuine saponins from soybean seeds**. *Bioscience, biotechnology, and biochemistry* 1993, **57**(4):546-550.

49. Hu J, Lee SO, Hendrich S, Murphy PA: **Quantification of the group B soyasaponins by high-performance liquid chromatography**. *JAgricFood Chem* 2002, **50**(9):2587-2594.

50. Tsujino Y, Tsurumi S, Yoshida Y, Niki E: **Antioxidative effects of dihydro-gamma-pyronyl-triterpenoid saponin (chromosaponin I)**. *Bioscience, biotechnology, and biochemistry* 1994, **58**(9):1731-1732.

51. Yoshiki Y, Kahara T, Okubo K, Sakabe T, Yamasaki T: **Superoxide-and 1, 1-diphenyl-2-picrylhydrazyl radical-scavenging activities of soyasaponin β g related to gallic acid**. *Bioscience, biotechnology, and biochemistry* 2001, **65**(10):2162-2165.

52. Yoshiki Y, Okubo K: **Active oxygen scavenging activity of DDMP (2, 3-dihydro-2, 5-dihydroxy-6-methyl-4H-pyran-4-one) saponin in soybean seed**. *Biosciences Biotechnology and Biochemistry* 1995, **59**(8):1556-1557.

53. Yoshikoshi M, Yoshiki Y, Okuba K, Seto J, Sasaki Y: **Prevention of hydrogen peroxide damage by soybean saponins to mouse fibroblasts**. *Planta Medica* 1996, **62**(Journal Article):252-255.

54. Lee SO, Simons AL, Murphy PA, Hendrich S: **Soyasaponins lowered plasma cholesterol and increased fecal bile acids in female golden Syrian hamsters**. *Experimental biology and medicine* 2005, **230**(7):472.

55. Oakenfull DG, Topping DL, Illman RJ, Fenwick DE: **Prevention of dietary hypercholesterolaemia in the rat by soya bean and quillaja saponins**. *Nutr Rep Int* 1984, **29**(Journal Article):1039-1041.

56. Ikeda T, Udayama M, Okawa M, Arao T, Kinjo J, Nohara T: **Partial hydrolysis of soyasaponin I and the hepatoprotective effects of the hydrolytic products. Study of the structure-hepatoprotective relationship of soyasapogenol B analogs**. *Chemical and Pharmaceutical Bulletin* 1998, **46**(2):359-361.

57. Kinjo J, Imagire M, Udayama M, Arao T, Nohara T: **Structure-hepatoprotective relationships study of soyasaponins I-IV having soyasapogenol B as aglycone**. *Planta Medica* 1998, **64**(Journal Article):233-236.

58. Miyao H, Arao T, Udayama M, Kinjo J, Nohara T: **Kaikasaponin III and Soyasaponin I, Major Triterpene Saponins of Abrus cantoneiensis, act on GOT and GPT: Influence of Transaminase Elevation of Rat Liver Cells Concomitantly Exposed to CCl~ 4 for One Hour**. *Planta Medica* 1998, **64**(Journal Article):5-7.

59. Hayashi K, Hayashi H, Hiraoka N, Ikeshiro Y: **Inhibitory activity of soyasaponin II on virus replication in vitro**. *Planta Medica* 1997, **63**(Journal Article):102-105.

60. Nakashima H, Okubo K, Honda Y, Tamura T, Matsuda S, Yamamoto N: **Inhibitory effect of glycosides like saponin from soybean on the infectivity of HIV in vitro**. *Aids* 1989, **3**(10):655.

61. Kinjo J, Nohara T: **Bioactive oleanene glucuronides obtained from fabaceous plants**. *Studies in Natural Products Chemistry* 2001, **25**(Journal Article):89-124.

62. Wenyan D, Dongping Z, Xueming G: **Enhancement Effect of Total Soyasaponin on Immune Function**. *Chinese Cereals and Oils Association* 2001(Journal Article).

63. Tsai CY, Chen YH, Chien YW, Huang WH, Lin SH: **Effect of soy saponin on the growth of human colon cancer cells**. *World Journal of Gastroenterology: WJG* 2010, **16**(27):3371.

64. Kim HY, Yu R, Kim JS, Kim YK, Sung MK: **Antiproliferative crude soy saponin extract modulates the expression of I [kappa] B [alpha], protein kinase C, and cyclooxygenase-2 in human colon cancer cells**. *Cancer letters* 2004, **210**(1):1-6.

65. Oh YJ, Sung MK: **Soybean saponins inhibit cell proliferation by suppressing PKC activation and induce differentiation of HT-29 human colon adenocarcinoma cells**. *Nutrition and cancer* 2001, **39**(1):132-138.

66. Rao AV, Sung MK: **Saponins as anticarcinogens**. *Journal of Nutrition* 1995, **125**(3 Suppl):717S.

67. Sung MK, Kendall CW, Koo MM, Rao AV: **Effect of soybean saponins and gypsophilla saponin on growth and viability of colon carcinoma cells in culture**. *Nutrition and cancer* 1995, **23**(3):259-270.

68. Sung MK, Kendall CWC, Rao AV: **Effect of soybean saponins and gypsophila saponin on morphology of colon carcinoma cells in culture**. *Food and Chemical Toxicology* 1995, **33**(5):357-366.

69. Bachran C, Bachran S, Sutherland M, Bachran D, Fuchs H: **Saponins in Tumor Therapy**. *Mini Reviews in Medicinal Chemistry* 2008, **8**(6):575-584.

70. Berhow MA, Wagner ED, Vaughn SF, Plewa MJ: **Characterization and antimutagenic activity of soybean saponins**. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 2000, **448**(1):11-22.

71. Ellington AA, Berhow M, Singletary KW: **Induction of macroautophagy in human colon cancer cells by soybean B-group triterpenoid saponins**. *Carcinogenesis* 2005, **26**(1):159.

72. Ellington AA, Berhow MA, Singletary KW: **Inhibition of Akt signaling and enhanced ERK1/2 activity are involved in induction of macroautophagy by triterpenoid B-group soyasaponins in colon cancer cells**. *Carcinogenesis* 2006, **27**(2):298.

73. Gurfinkel DM, Rao AV: **Soyasaponins: the relationship between chemical structure and colon anticarcinogenic activity**. *Nutrition and cancer* 2003, **47**(1):24-33.

74. Hu J, Reddy MB, Hendrich S, Murphy PA: **Soyasaponin I and Sapongenol B Have Limited Absorption by Caco-2 Intestinal Cells and Limited Bioavailability in Women 1**. *Journal of Nutrition* 2004, **134**(8):1867-1873.

75. Hu J, Zheng YL, Hyde W, Hendrich S, Murphy PA: **Human fecal metabolism of soyasaponin I**. *JAgricFood Chem* 2004, **52**(9):2689-2696.

76. Koratkar R, Rao AV: **Effect of soya bean saponins on azoxymethane-induced preneoplastic lesions in the colon of mice**. *Nutrition and cancer* 1997, **27**(2):206-209.

77. MacDonald RS, Guo JY, Copeland J, Browning Jr JD, Sleper D, Rottinghaus GE, Berhow MA: **Environmental influences on isoflavones and saponins in soybeans and their role in colon cancer**. *Journal of nutrition* 2005, **135**(5):1239.

78. Thiagarajan DG, Bennink MR, Bourquin LD, Kavas FA: **Prevention of precancerous colonic lesions in rats by soy flakes, soy flour, genistein, and calcium**. *American Journal of Clinical Nutrition* 1998, **68**(6):1394S.

79. Hakkak R, Korourian S, Ronis MJJ, Johnston JM, Badger TM: **Soy protein isolate consumption protects against azoxymethane-induced colon tumors in male rats**. *Cancer letters* 2001, **166**(1):27-32.

80. Rao CV, Wang CX, Simi B, Lubet R, Kelloff G, Steele V, Reddy BS: **Enhancement of experimental colon cancer by genistein**. *Cancer research* 1997, **57**(17):3717.

81. Sidhu GS, Oakenfull DG: **A mechanism for the hypocholesterolaemic activity of saponins**. *British journal of nutrition* 2007, **55**(03):643-649.

82. Oakenfull D, Sidhu GS: **Could saponins be a useful treatment for hypercholesterolaemia?** *European journal of clinical nutrition* 1990, **44**(1):79-88.

83. Oakenfull DG: **Aggregation of saponins and bile acids in aqueous solution**. *AustJChem* 1986, **39**(10):1671-1683.

84. Potter JD, Illman RJ, Calvert GD, Oakenfull DG, Topping DL: **Soya saponins, plasma lipids, lipoproteins and fecal bile acids: a double blind cross-over study**. *NutrRepInt* 1980, **22**(Journal Article):521-528.

85. Nagengast FM, Grubben M, Van Munster IP: **Role of bile acids in colorectal carcinogenesis**. *European journal of cancer* 1995, **31**(7-8):1067-1070.

86. Kanagalingam K, Strause E: **Influence of lithocholic acid on 2-aminoanthracene-induced alterations in DNA synthesis in rat tissues in vivo**. *Cancer biochemistry biophysics* 1980, **4**(3):159-166.

87. Kulkarni MS, Heidepriem PM, Yielding KL: **Production by lithocholic acid of DNA strand breaks in L1210 cells**. *Cancer research* 1980, **40**(8 Part 1):2666.

88. Rafter JJ, Eng VW, Furrer R, Medline A, Bruce WR: **Effects of calcium and pH on the mucosal damage produced by deoxycholic acid in the rat colon**. *Gut* 1986, **27**(11):1320.

89. Nishizuka Y: **The role of protein kinase C in cell surface signal transduction and tumour promotion**. 1984(Journal Article).

90. Craven PA, Pfanstiel J, DeRubertis FR: **Role of activation of protein kinase C in the stimulation of colonic epithelial proliferation and reactive oxygen formation by bile acids**. *Journal of Clinical Investigation* 1987, **79**(2):532.

91. Lapre JA, Van der Meer R: **Diet-induced increase of colonic bile acids stimulates lytic activity of fecal water and proliferation of colonic cells**. *Carcinogenesis* 1992, **13**(1):41.

92. Vahouny GV, Satchithanandam S, Lightfoot F, Grau L, Haas-Smith S, Kritchevsky D, Cassidy MM: **Morphological disruption of colonic mucosa by free or cholestyramine-bound bile acids**. *Digestive diseases and sciences* 1984, **29**(5):432-442.

93. Van Munster IP, Tangerman A, De Haan AFJ, Nagengast FM: **A new method for the determination of the cytotoxicity of bile acids and aqueous phase of stool: the effect of calcium**. *European journal of clinical investigation* 1993, **23**(12):773-777.

94. Gestetner B, Birk Y, Tencer Y: **Soybean saponins. Fate of ingested soybean saponins and the physiological aspect of their hemolytic activity**. *Journal of Agricultural and Food Chemistry* 1968, **16**(6):1031-1035.

95. Yoshikoshi M, Kahara T, Yoshiki Y, Furukawa Y, Okubo K, Amarowicz R, Ito M: **Metabolism and nonabsorption of soybean hypocotyl saponins in the rat model**. *Acta Alimentaria* 1995, **24**(4):355-364.

96. Malinow MR, McNulty WP, McLaughlin P, Stafford C, Burns AK, Livingston AL, Kohler GO: **The toxicity of alfalfa saponins in rats**. *Food and cosmetics toxicology* 1981, **19**(Journal Article):443-445.

97. Ishaaya I, Birk Y, Bondi A, Tencer Y: **Soyabean saponins IX.-Studies of their effect on birds, mammals and cold-blooded organisms**. *Journal of the science of food and agriculture* 1969, **20**(7):433-436.

98. Murphy PA, Hu J, Barua K, Hauck CC: **Group B Saponins in Soy Products in the US Department of Agriculture Iowa State University Isoflavone Database and Their Comparison with Isoflavone Contents**. *JAgricFood Chem* 2008, **56**(18):8534-8540.

99. Wei Zhang SPT, David G. Popovich: **Generation of Group B Soyasaponins I and III by Hydrolysis**. *JAgricFood Chem* 2009(57):3620-3625.